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(54) Title: PRODUCTION OF BIOLOGICAL MATERIALS BY SIMULTANEOUS AEROBIC AND ANAEROBIC RESPIRATION

(57) **Abstract**

A process for the production of biological products by microorganisms comprising the steps of: selecting a microorganism that is capable of utilizing oxygen or an alternative oxidant source other than oxygen for cellular respiration; providing a culture medium suitable for the growth of the microorganism, wherein the medium comprises at least one carbon source; inoculating the culture medium with a desired cellular concentration of the microorganism; aerating the culture medium with oxygen, wherein the process has a maximum oxygen supply rate to the culture medium; supplying the culture medium with an alternative oxidant source, other than oxygen, such that when the cellular respiration requirements of the microorganisms within the culture medium is less than the maximum rate of oxygen supply to the culture medium, then the microorganisms will substantially utilize oxygen for cellular respiration, and when the oxygen requirements for cellular respiration of the microorganisms within the culture medium is greater than the maximum rate of oxygen supply to the culture medium, then a portion of the microorganisms within the culture medium will utilize the alternative oxidant source for cellular respiration; maintaining the culture medium at a desired pH and temperature; and allowing the culture medium to incubate for a time sufficient to produce a desired quantity of a biological product. The invention also provides a process for increasing concentration of microorganisms in a defined culture medium.

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PRODUCTION OF BIOLOGICAL MATERIALS BY SIMULTANEOUS AEROBIC AND ANAEROBIC RESPIRATION

CROSS REFERENCE TO RELATED APPLICATIONS

The present application claims priority from United States Provisional Patent Application No. 60/108,837, filed on November 18, 1998.

TECHNICAL FIELD OF THE INVENTION

The present invention relates to the production of biological materials by microorganisms. The present invention more particularly relates to a process for the preparation of biological products, such as biosurfactants, by microorganisms under simultaneous aerobic and anaerobic respiring conditions.

BACKGROUND OF THE INVENTION

Cells are the real workers in biological processes. To increase process productivity, it is desirable to grow the cells to concentrations as high as possible. For aerobic biological processes, the cell concentrations employable are most commonly limited by the rate of oxygen transfer to the cell population that is achievable by a particular process. Therefore, the productivity of biological materials by aerobic fermentation processes is directly limited by the oxygen supply to the cells.

It is well known that oxygen gas is only slightly soluble in aqueous media and, therefore the supply of oxygen must be replenished by inefficient mass transfer across the gas/liquid interface. This is traditionally achieved by vigorous aeration and/or

agitation to promote the interfacial transfer of oxygen from gas bubbles to the aqueous media.

However, this limitation is especially serious in biological production processes that prevent the use of vigorous agitation or aeration to promote the interfacial transfer of oxygen gas in the aqueous media, such as in the production of highly foaming biosurfactants such as rhamnolipids, highly viscous biopolymers such as xanthan gum, oxygen sensitive products, and production of biological materials, such as antibiotics, by shear-sensitive organisms.

It is widely known in the art to produce biosurfactants, such as rhamnolipids, by conventional aerobic fermentation processes. For example, United States Patent No. 5,501,966 to Giani et al discloses a method for the biotechnological preparation of L-rhamnose by microorganisms, such as *Pseudomonas aeruginosa*. The bacterial strain *Pseudomonas aeruginosa* is used to secrete rhamnolipids, under aerobic fermentation conditions, into the culture supernatant. The *Pseudomonas aeruginosa* is fermented in a medium containing vegetable oils, such as olive, corn and sunflower oil, as the carbon source. Aeration is employed using sterile air to provide oxygen to the fermentation solution. The reference discloses that it is necessary to add a suitable anti-foaming agent to the fermentation solution during the fermentation process. The L-rhamnose is recovered directly from the culture solution by hydrolysis of the rhamnolipids, without separation of the cell material and without isolation of the rhamnolipids before hydrolysis.

Furthermore, United States Patent No. 4,628,030 to Kaeppeli et al discloses a method for the production of rhamnolipids by the microorganism *Pseudomonas aeruginosa*. According to the reference, rhamnolipids are produced by the cultivation of rhamnolipid producing microorganisms of the genus *Pseudomonas* in an aqueous

culture medium suitable for the growth of the microorganism. The microorganism is cultured in a continuous submerged culture under aerobic conditions and with a continuous supply of fresh culture medium, and continuous removal of a solution of partially spent culture medium and produced rhamnolipids; and limiting the amount of at least two essential growth substances selected from the group consisting of carbon, nitrogen, sulfur, phosphorous, sodium, potassium, magnesium, calcium, iron, zinc, manganese, boron, cobalt, copper and molybdenum, in the culture medium such that the quantity of essential growth substance in the partially spent culture medium is less than half of the amount in the fresh culture medium.

United States Patent No. 4,814,272 to Wagner et al discloses a method for the microbiological production of rhamnolipids comprising culturing the microorganism *Pseudomonas* species 2874 under aerobic conditions in an aqueous nutrient solution containing at least one carbon source at a pH value of 6.5 to 7.3 and a temperature of 30° to 37°C. The aqueous culture is either extracted directly with a suitable solvent and evaporated, or the resulting wet cell mass is separated from the culture broth and incubated with a carbon source to further increase rhamnolipid production.

United States Patent No. 4,933,281 to Daniels et al discloses a method for producing rhamnose comprising the steps of growing the microorganism *Pseudomonas* in a defined culture medium containing vegetable oil to produce rhamnolipids; hydrolyzing the rhamnolipids to form rhamnose and 3-hydroxydecanoic acid; and separating the rhamnose from the acid. During fermentation, sterile air is sparged into the fermentor at a rate of 0.1 to 1.0 VVM (volume air per volume fermentor liquid per minute), with a rate of 0.5 VVM being most perferred.

In addition to biosurfactant production, it is also known to produce viscous biopolymers, such as xanthan gum, by conventional aerobic fermentation processes.

United States Patent No. 4,352,882 to Maury discloses a method for production of a polysaccharide gum, such as xanthan gum, by microemulsion comprising inoculating an aqueous culture medium comprising a carbohydrate source and a nitrogen source with a polysaccharide gum-producing microorganism, mechanically agitating and aerating the culture medium under aerobic conditions to effect fermentation thereof, wherein the culture medium is dispersed in about 20 to 80% of its weight of a water insoluble oil in which the resultant polysaccharide is also insoluble. The reference further teaches that the oil in the microemulsion significantly increases the oxygen transfer efficiency leading to an increased rate of reaction.

The above referenced conventional methods of biological production of biosurfactants, including rhamnolipids, have serious disadvantages. The most significant technical problem associated with the above referenced methods of rhamnolipid production under aerobic conditions is the extensive formation of foam. Due to the rapid foam formation and high foam stability, the elimination of foam during biological processes continues to be a problem.

There have been numerous attempts to utilize chemical anti-foam agents to eliminate foam formation during aerobic production of biosurfactants. However, the known anti-foam agents are very expensive, and may affect cell metabolism, downstream product recovery and purification, and wastewater processing.

There have also been attempts to control the foaming associated with aerobic fermentation in biological processes through the use of a mechanical apparatus that is in fluid communication with the fermentation tank. For example, United States Patent No. 5,476,573 to Hirose et al. discloses an apparatus for defoaming and controlling aerobic culture fermentation comprising a first means for separating vapor from liquid of a foam; a second means for separating residual liquid of said vapor received from

said first means for separating, in fluid communication with said first means for separating; a means for recirculating liquid from said first means for separating and condensed residual liquid from said second means for separating, said means for recirculating being in fluid communication with said first means for separating and said second means for separating, and a sensor for detecting foams, located between and in fluid communication with said first means for separating and said second means for separating. The reference further discloses that an optional defoaming device may be included, which may be based on either a rotary body rotating at a high speed by use of an electric motor which beat the foams, or on a centrifugal atomizer.

It is, therefore, desirable to develop a process for production of biological materials to avoid the problems associated with oxygen limitation and foam formation arising from continuous aeration and vigorous agitation that is required during known biological production processes.

In addition, with the technological advancements in the area of genetic engineering of cells, it is desirable to develop a process for the production of biological materials, that can employ genetically engineered or manipulated host cells, which avoids the problems associated with oxygen limitation and foam formation arising from continuous aeration and vigorous agitation that is required during known biological production processes.

Heretofore, the prior art has not taught to produce biological materials, such as biosurfactants, viscous biopolymers, oxygen sensitive products, and the like by simultaneous aerobic and anaerobic respiration processes.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a method for the production of biological materials that eliminates the problems associated with oxygen limitation encountered in solely aerobic bioprocesses.

It is another object of the present invention to provide a method for production of biological materials that eliminates foam formation problems associated with solely aerobic production of biological materials.

It is another object of the present invention to provide a method for production of biological materials that allows use of high cell concentrations.

It is another object of the present invention to provide a method for production of biological materials that increases volumetric productivity of biological products.

It is another object of the present invention to provide a method for production of biological materials that reduces the cost of downstream recovery and purification of the final biological products.

It is another object of the present invention to provide a method to increase production of biological materials that are oxygen sensitive.

It is another object of the present invention to provide a method to produce biological materials that can utilize or employ genetically engineered or manipulated microorganisms or cells.

The foregoing objects, together with the advantages thereof over the known art relating to aerobic production of biological materials, which shall become apparent from the specification which follows, are accomplished by the invention as hereinafter described and claimed.

The present invention, therefore, provides a process for the production of biological products by microorganisms comprising the steps of: selecting a microorganism that is capable of utilizing oxygen or an alternative oxidant source other than oxygen for cellular respiration; providing a culture medium suitable for the growth of the microorganism, wherein the medium comprises at least one carbon source; inoculating the culture medium with a desired cellular concentration of the microorganism; aerating the culture medium with oxygen, wherein the process has a maximum oxygen supply rate to the culture medium; supplying the culture medium with an alternative oxidant source, other than oxygen, such that when the cellular respiration requirements of the microorganisms within the culture medium is less than the maximum rate of oxygen supply to the culture medium, then the microorganisms will substantially utilize oxygen for cellular respiration, and when the cellular respiration requirements of the microorganisms within the culture medium is greater than the maximum rate of oxygen supply to the culture medium, then a portion of the microorganisms will utilize the available oxygen within the medium and another portion of the microorganisms within the culture medium will simultaneously utilize the alternative oxidant source for cellular respiration; maintaining the culture medium at a desired pH and temperature; and allowing the culture medium to incubate for a time sufficient to produce a desired quantity of a biological product.

The present invention also provides a process for the preparation of biological products under anaerobic respiring conditions comprising: selecting a microorganism that is capable of utilizing an alternative oxidant source other than oxygen for cellular

respiration under anaerobic conditions; providing a culture medium suitable for the growth of the microorganism, wherein the medium comprises at least one carbon source; inoculating the culture medium with a desired cellular concentration of the microorganism; supplying an alternative oxidant source other than oxygen to the culture medium; maintaining the culture medium at a desired pH and temperature; and allowing the culture medium to incubate for a time sufficient to produce a desired quantity of a biological product.

The present invention also provides a process for creating an increased concentration of microorganisms in a defined medium comprising the steps of: selecting a microorganism that is capable of utilizing oxygen or an alternative oxidant source other than oxygen for cellular respiration; providing a culture medium suitable for the growth of the microorganism, wherein the medium comprises at least one carbon source; inoculating the culture medium with a desired cellular concentration of the microorganism; aerating the culture medium with oxygen, wherein the process has a maximum oxygen supply rate to the culture medium; supplying the culture medium with an alternative oxidant source, other than oxygen, such that when the cellular respiration requirements of the microorganisms within the culture medium is less than the maximum rate of oxygen supply to the culture medium, then the microorganisms will substantially utilize oxygen for cellular respiration, and when the oxygen requirements for cellular respiration of the microorganisms within the culture medium is greater than the maximum rate of oxygen supply to the culture medium, then a portion of the microorganisms will utilize the oxygen available within the medium and another portion of the microorganisms within the culture medium will simultaneously utilize the alternative oxidant source for cellular respiration; maintaining the culture medium at a desired pH and temperature; and allowing the culture medium to incubate for a time sufficient to produce a desired concentration of microorganisms within the culture medium.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing the growth of *Pseudomonas aeruginosa* on various carbon substrates under anaerobic denitrification conditions.

Figure 2 is a graph showing the growth of *Pseudomonas aeruginosa* over time in aqueous culture media containing an initial addition of rhamnolipids as compared to growth in an aqueous medium without rhamnolipids. Cell growth is measured by the increase in cell protein (g/L).

Figure 3 is a graph showing *Pseudomonas aeruginosa* growth on a glycerol substrate under anaerobic denitrifying conditions.

Figure 4 is a graph showing *Pseudamonas aeruginosa* growth on a palmitic acid substrate under anaerobic denitrifying conditions.

Figure 5 is a graph showing *Pseudomonas aeruginosa* growth on a stearic acid substrate under anaerobic denitrifying conditions.

Figure 6 is a graph showing rhamnolipid production by *P. aeruginosa* under anaerobic denitrifying conditions followed by fermentation under aerobic conditions, using palmitic acid as the carbon source.

DETAILED DESCRIPTION OF THE INVENTION

It is now been discovered that a method utilizing both aerobic and anaerobic respiration can be used to produce high cell concentrations within a culture medium, which results in an increase in the volumetric productivity of biological products, such

as surfactants and viscous biopolymers. It is envisioned that the method of the present invention can be useful as a batch or continuous process for the production of biological materials. The process of the present invention is especially useful in the production of biosurfactants, such as rhamnolipids. Rhamnolipids are anionic extracellular biosurfactants that are useful in many commercial applications in the petroleum, pharmaceutical and food processing industries. The present invention is premised on the fact that various species of microorganisms can use certain alternative oxidants, such as nitrates and the like, other than molecular oxygen for purposes of metabolic or cellular respiration to avoid problems associated with oxygen limitation in bioprocesses. By using alternative oxidant sources, the serious limitations associated with oxygen supply to the cells, such as reduced cell number and foam generation can be eliminated.

The present invention provides a process for the production of biological products by microorganisms. A suitable microorganism that is capable of undergoing or utilizing anaerobic respiration must be selected. A defined culture medium must be provided that is suitable for the growth of the microorganism to carry out the biological processes. The medium comprises at least one carbon source for the microorganism. Once a suitable culture medium has been selected, a desired cellular concentration or quantity of microorganism is introduced or added to the culture medium. The culture medium is aerated with oxygen and also supplied with an alternative oxidant source.

It should be noted that the process has a maximum oxygen transfer or supply rate into the culture medium. When the cellular respiration requirements (the oxygen requirements) of the microorganisms within the culture medium is less than the maximum rate of oxygen transfer or supply into the culture medium, then the microorganisms will utilize the oxygen within the culture medium for cellular respiration. However, as the concentration of cells within the medium begins to

increase, the cellular respiration requirements, and consequently the oxygen consumption, of the microorganisms within the culture medium increases. When the cellular respiration requirements of the microorganisms within the culture medium becomes greater than the maximum oxygen transfer or supply rate into the culture medium, a portion of the microorganisms will utilize the available oxygen within the medium, and another portion of the microorganisms within the medium will simultaneously begin to utilize the alternative oxidant source for cellular respiration requirements. This process, therefore, enables the microorganisms to reach high concentrations within the medium and remain viable throughout productions of biological materials. With a portion of the microorganisms using the alternative oxidant source for anaerobic respiration, cell growth continues and the concentration of cells within the medium reaches concentrations that would otherwise be impossible due to oxygen limitation.

The culture medium is maintained at a desired pH and temperature, and the culture medium is allowed to incubate for a time sufficient to produce a desired quantity of a biological product. The resulting biological product is isolated and recovered from the culture medium.

The microorganisms that are useful in the present invention are those selected from bacteria, yeast, molds, archaea, and the like. Preferred microorganisms are facultative aerobic bacteria and obligate anaerobic bacteria.

Facultative aerobic bacteria are those species of bacteria that can either utilize oxygen for respiration purposes under aerobic conditions, or can utilize alternative oxidants other than oxygen for respiration purposes in the absence of oxygen. Suitable species of facultative aerobic bacteria that may be used in the present invention include, but are not limited to, nitrate/nitrite respiration bacteria such as *Pseudomonas*

aeruginosa, Pseudomonas fluorescens, Paracoccus denitrificans, Micrococcus halodenitrificans, Klebsiella aerogenes, Escherichia coli, and the like; hyperthermophilic Archaea bacteria such as *Acidianus*; and the fumarate respiration bacteria such as *Wolinella succinogenes, Desulfovibrio gigas, Clostridia, Escherichia coli* and *Proteus rettgeri*. A preferred facultative aerobic bacterium is that of the genus *Pseudomonas*.

According to the present invention the quantity or concentration of the microorganism that is added to the culture medium for processes employing growing cells is preferably from about 0.1 g/L to about 10 g/L, more preferably from about 0.5 g/L to about 5 g/L. For processes employing non-growing (stationary phase) cells, the quantity or concentration of the microorganism that is added to the culture medium is preferably from about 5 g/L to about 50 g/L.

The production of biological products according to the method of the present invention requires that a defined liquid culture medium be selected. The liquid culture medium contains at least one carbon source (substrate) for production of biological products. The liquid culture medium used in the present invention can be any known culture medium that comprises nutrients that can support cellular growth, particularly microbial growth. Without limiting the processes of the present invention to any particular culture medium, a representative liquid culture medium formulation may comprise the following mineral substituents: 4 g/liter of NH_4Cl, 11 g/liter of K_2HPO_4, 0.5 g/liter NaCl, 0.3 g/liter $MgSO_4 \cdot 7H_2O$, 0.03 g/liter $FeCl_3 \cdot 6H_2O$, 0.01 g/liter $CaCl_2$, and 0.01 g/liter $MnCl_2 \cdot 4H_2O$.

Carbon sources useful in the present invention include, but are not limited to, fatty acids; glycerol; low molecular weight organic acids such as malate, acetate,

pyruvate and the like; vegetable oil; yeast extract; peptone; and carbohydrates such as glucose.

Suitable fatty acids that can be utilized in the present invention include, but are not limited to, fatty acids such as palmitic acid, stearic acid, oleic acid, linoleic acid, arachidic acid, butyric acid, caproic acid, lauric acid, linolenic acid, and the like. Palmitic acid is a preferred fatty acid that may be utilized in the processes of the present invention.

Vegetable oils, such as corn oil, peanut oil, coconut oil, linseed oil, olive oil, soy bean oil, sunflower oil, and the like, may be used as the carbon substrate in the present invention. A preferred vegetable oil for use in the present invention is corn oil.

As described hereinabove, the culture medium is simultaneously aerated with oxygen and supplied with an alternative oxidant source. The population of microorganisms in the culture medium that are not utilizing oxygen for cellular respiration will utilize an alternative oxidant present in the medium instead of oxygen as the final electron acceptor in the cellular respiratory chain. The term "oxidant", as used throughout the specification, refers to the molecules or compounds that can serve as the terminal electron acceptor in the respiratory chain of a cell. According to the present invention, suitable alternative oxidants are selected from nitrates, nitrites, sulfates, sulfites, carbon dioxide or carbonates, bicarbonates, fumarates, sulfur, manganic ion, ferric ion, selenate, dimethyl sulfoxide, arsenate, trimethylamine-N-oxide and glycine.

According to the present invention suitable nitrates include, but are not limited to, those nitrates selected from sodium nitrate, potassium nitrate, calcium nitrate,

magnesium nitrate, ammonium nitrate, nitric acid, and the like. A preferred nitrate for use as the alternative oxidant source is sodium nitrate.

According to the present invention suitable nitrites include, but are not limited to, those nitrites selected from sodium nitrite, potassium nitrite, calcium nitrite, ammonium nitrites, nitrous acid and the like. A preferred nitrite for use as the alternative oxidant source is sodium nitrite.

According to the present invention suitable sulfates include, but are not limited to, those sulfates that are selected from sodium sulfate, potassium sulfate, calcium sulfate, iron sulfate, magnesium sulfate, ammonium sulfate, zinc sulfate, copper sulfate, cobalt sulfate, manganese sulfate, dilute sulfuric acid, and the like.

According to the present invention suitable sulfites include, but are not limited to, those sulfites that are selected from calcium sulfite, sodium sulfite, potassium sulfite, iron sulfite, magnesium sulfite, ammonium sulfite, zinc sulfite, copper sulfite, cobalt sulfite, manganese sulfite, and the like.

According to the present invention suitable carbonates and bicarbonates include, but are not limited to, those carbonates and bicarbonates that are selected from calcium carbonate, sodium carbonate, potassium carbonate, calcium bicarbonate, sodium bicarbonate, potassium bicarbonate, carboxylic acid, and the like.

Suitable fumarates useful in the present invention include, but are not limited to, those fumarates that are selected from the group consisting of disodium fumarate ($C_4H_2O_4Na_2$), sodium fumarate ($C_4H_3O_4Na$), dipotassium fumarate ($C_4H_2O_4K_2$), potassium fumarate ($C_4H_3O_4K$), fumaric acid ($C_4H_4O_4$), and the like.

Depending on the microorganism employed, the alternative oxidant is maintained in the culture medium at a concentration from about 0.01 g/L to about 10 g/L preferably at a concentration from about 0.05 g/L to about 5 g/L, and more preferably from about 0.1 g/L to about 0.5 g/L.

According to the present invention, a desired amount of a surfactant may also be added to the culture medium to facilitate the mass transfer of the otherwise poorly soluble or insoluble carbon substrate into the culture medium. The addition of the surfactant to the culture medium facilitates the dispersion or solubilization of the carbon source into the culture medium. Furthermore, the addition of the surfactant to the culture medium assists in the penetration of the carbon source through the cell wall of the microorganism. The amount of surfactant to be added to the culture medium is from about 0.01 g/L to about 10 g/L, preferably from about 0.05 g/L to about 1 g/L, and most preferably from about 0.1 g/L to about 0.5 g/L.

The microorganism is incubated with the carbon substrate contained in the culture medium for a time sufficient at a desired temperature and pH to produce a desired quantity of a biological product. The temperature of the culture medium influences the growth and the survival of the microorganism employed. For every organism there is minimum temperature below which growth no longer occurs, an optimum temperature at which growth is most rapid, and a maximum temperature above which growth falls sharply to zero. Many microorganisms have temperature ranges as low as about 5°C to about 10°C, while some microorganisms have optimum temperatures greater than about 100°C. According to their temperature optima, microorganisms are classified into psychrophiles, having temperature optima of less than about 10°C, mesophiles, having temperature optima from about 15°C to about 45°C, thermophiles, having temperature optima of greater than about 45°C, and hyperthermophiles having temperature optima of greater than about 80°C. Therefore,

the temperature of the culture medium throughout the incubation period is dependant on the microorganism selected. For example, the temperature of the culture medium for *P. aeruginosa* during incubation is preferably carried out in a temperature range of about 20°C to about 40 °C, more preferably in a temperature range of about 27°C to about 38 °C, and most preferably between about 30°C and about 37°C.

Throughout the incubation period, the pH of the culture medium is maintained in an optimal pH range, which is dependent on the species of microorganism chosen.

In another embodiment, the present invention provides a process for the production of biosurfactants, such as rhamnolipids, by the facultative aerobic bacterium, *Pseudomonas aeruginosa*. In the absence of oxygen, *Pseudomonas aeruginosa* within the culture medium will utilize an alternative oxidant source, such as sodium nitrate, for cellular respiration purposes. It has been found that limiting the essential growth nutrient phosphorous from the culture media brings about the onset of the stationary phase, and facilitates increased rhamnolipid production by *Pseudomonas aeruginosa*.

The temperature range for the production of rhamnolipids by *Pseudomonas aeruginosa* by anaerobic denitrification is from about 20 to about 40°C, more preferably from about 27 to about 38°C, and most preferably from about 30 to about 37°C. The pH range of the culture medium for the production of biosurfactants by *Pseudomonas aeruginosa* is optimally from about 6 to about 7, more preferably between about 6.5 to about 6.8.

It should be noted that the process of the present invention can utilize genetically engineered or manipulated host microorganisms or cells for production of biological materials, provided that the genetically engineered host cell is capable

utilizing an alternative oxidant source. The process, including the selection of a suitable culture medium, carbon substrate, alternative oxidants and reaction conditions, is essentially the same as disclosed hereinabove, but employs genetically engineered microorganism. A DNA sequence encoding for a desired biological product is selected. A suitable host microorganism that is capable of undergoing anaerobic respiration is transfected with the DNA sequence, and is added to a suitable culture medium.

In another embodiment of the present invention, production of biological products under anaerobic conditions is provided. A culture medium suitable for the growth of said microorganism, and comprising at least one carbon source is provided. The culture medium is inoculated with a desired cellular concentration of the microorganism. An oxidant other than oxygen is supplied to the culture medium, under anaerobic conditions and in the absence of oxygen. The culture medium is maintained at a desired pH and temperature, and allowed to incubate for a time sufficient to produce a desired quantity of a biological product. An essential cellular growth nutrient may be substantially limited from the culture medium to inhibit cell growth and facilitate to the production of biological product.

In addition to the facultative aerobes described hereinabove, obligate anaerobes can be employed as the microorganism in this embodiment. Preferred obligate anaerobes are obligate anaerobic bacteria.

Obligate anaerobic bacteria are those species of bacteria that can only survive and grow under anaerobic conditions, that is, in the complete absence of oxygen. Suitable species of obligate anaerobic bacteria that may be used in the present invention include, but are not limited to, the homoacetogenic and methoanogenic Archaea bacteria capable of carbon dioxide/carbonate respiration; the sulfate-respiration bacteria

such as *Desulfovibrio, Desulfomonas, Desulfotomaculum, Desulfobulbus, Desulfococcus, Desulfobacter, Desulfosarcine, Desulfonema*, and the like; the sulfur-respiration bacteria such as *Desulfurmonas*; hyperthermophilic Archaea bacteria, such as *Thermoproteus, Pyrococcus, Thermococcus*, and the like; and ferric ion (Fe^{3+}) respiration bacteria such as *Shewanella putrefaciens*.

An essential growth nutrient may be limited from the culture medium in order to regulate cellular growth and to reach the resting or stationary phase. An essential growth nutrient that can be excluded or removed from the liquid culture media is selected from sulfur, phosphorous, nitrogen, magnesium, calcium and iron. The terms "resting phase" and "stationary phase", as used throughout the specification, refer to the phases when the cells are not undergoing cellular division.

Examples of biological products that can be produced according to the methods of the present invention, but are not limited to, biosurfactants, viscous biopolymers, proteins, enzymes, specialty chemicals, oxygen sensitive products, and products produced by shear sensitive microorganisms.

The biosurfactants that can be produced according to the methods of the present invention include, but are not limited to, rhamnolipids, sophorolipids, trehalose mycolates, trehalose esters, monosaccharide mycolates, disaccharide mycolates, trisaccharide mycolates, phospholipids, fatty acids, gramicidens, polymyxins, omithine-lipid, cerilipin, lysin-lipid, surfactin, subtilisin, peptide-lipid, heteropolysaccharide, lipoheteropolysaccharide, poly-saccharide-protein, manno-protein, carbohydrate-protein, mannan-lipid complex, mannose/erythrose-lipid, carbohydrate-protein-lipid-complex and fimbriae.

The viscous biopolymers that can be produced according to the methods of the present invention include, but are not limited to, xantham gum, pullulan, dextran and polyalginic acid.

The products produced according to the process of the present invention by shear sensitive microorganisms are selected from the group consisting of antibiotics, enzymes, cellulases, amylase, proteases, liginases, and organic acids.

GENERAL EXPERIMENTAL

The following example of rhamnolipid production by *P. aeruginosa* is set forth to illustrate the methods of the present invention. However, the examples should not be construed as limiting the present invention in any manner.

The production of rhamnolipid biosurfactants under phosphorous-limited denitrifying anaerobic conditions was evaluated. The experiment was conducted in a 2 liter Erlenmeyer flask, having a 600 milliliter working volume. The experiments were conducted at a temperature of about 23°C, and the pH of the working volume was maintained at 6.5 $\pm$ 0.1 by automatic pH control with 1N HNO_3 and 0.5N NaOH.

P. aeruginosa was added to the medium to form a culture. Sodium nitrate ($NaNO_3$) was included in the initial culture medium at a concentration of 0.5 g/liter of NO_3^--N. The sodium nitrate was added periodically throughout the test period to maintain the concentration of NO_3^--N at about 0.1 to about 0.5 g/liter. 16 g/liter of palmitic acid was added to the culture medium as the carbon substrate. The culture medium was allowed to incubate for about 500 hours at the experimental conditions described hereinabove.

The results demonstrate that rhamnolipids can be produced under anaerobic denitrification conditions, without the problems of foaming and oxygen limitation. The present invention provides a process for the production of biological products, wherein a desired amount of a surfactant is added to the culture medium to facilitate the mass transfer of the otherwise poorly soluble or insoluble carbon substrate into the culture medium. The culture is allowed to incubate for a time sufficient to produce a desired quantity of a biological product.

It has been found that the addition of the surfactant to the culture medium facilitates the dispersion or solubilization of the carbon source into the culture medium. Furthermore, the addition of the surfactant to the culture medium assists in the penetration of the carbon source through the cell wall of the microorganism. The amount of surfactant to be added to the culture medium is from about 0.01 g/L to about 10 g/L, preferably from about 0.05 g/L to about 1 g/L, and most preferably from about 0.1 g/L to about 0.5 g/L. The results indicate that rhamnolipids are produced by *Pseudomonas aeruginosa* under denitrifying anaerobic conditions. The rate of rhamnolipid production by anaerobic denitrification is about 2 milligrams of rhamnolipids/gram of cell protein/hour.

Biosurfactants, such as rhamnolipids, are extremely effective in emulsifying and solubilizing hydrocarbons and, therefore, are quite useful in oil recovery processes and mobilizing non-aqueous phase liquid contaminants in soils and aquifiers. Rhamnolipids, because of their antibacterial, antiviral, antifungal, and mycoplasmacidal properties, also find potential use in the pesticide applications. In addition, rhamnolipids have been implicated as an additive to concrete formulations to increase the strength of concrete, thus reducing the potential for concrete damage.

Rhamnolipids have particular application in industrial petroleum processes, including emulsification and demulsification, separation, formation of low viscosity emulsion products to transport heavy crudes, emulsion washing, formation of slurries, corrosion inhibition, enhancement of oil recovery and promotion of hydrocarbon biodegradation.

Biosurfactants, such as rhamnolipids, are particularly useful in the cosmetic or personal hygiene industry, because of their low toxicity, excellent moisturizing properties and compatibility with mammalian skin.

The rhamnolipids produced according to the method of the present invention can also be used as a source of rhamnose sugar. The isolated rhamnolipids are hydrolyzed to produce a mixture of rhamnose sugar and beta-hydroxydecanoic acid. The rhamnose is easily separated from the beta-hydroxydecanoic acid. The rhamnose can be used as a fine chemical in many industrial and scientific applications.

According to the present invention, much larger cell concentrations may be employed to give a higher volumetric productivity and product concentrations for more economical product recovery and purification.

The use of the methods of the present invention in biological processes effectively circumvents the limitations of oxygen supply and foam-generation problems that are traditionally associated with aerobic production of biological products such as biosurfactants.

The extremely soluble alternative oxidants can be easily supplied to meet the respiration needs of high cell concentrations and, consequently, achieve very high

process productivity, without the need for vigorous agitation of the culture medium within the reactor.

It has also been demonstrated that the utilization of the methods of the present invention in biological processes is beneficial to the production of biological materials that are sensitive to the presence of molecular oxygen.

Based upon the foregoing disclosure and description, it should now be apparent that the use of the described methods herein will carry out the objects set forth hereinabove. It is, therefore, to be understood that any variations evident fall within the scope of the claimed invention, and the selection of specific carbon sources, culture media, alternate oxidant sources, limiting nutrients, pH and temperature conditions, and selection of microorganism can be determined without departing from the spirit of the invention herein disclosed and described. Thus, the scope of the invention shall include all modifications and variations that may fall within the scope of the claims.

WE CLAIM:

1. A process for the production of biological products by microorganisms comprising the steps of:

selecting a microorganism that is capable of utilizing oxygen or an alternative oxidant source other than oxygen for cellular respiration;

providing a culture medium suitable for the growth of the microorganism, wherein the medium comprises at least one carbon source;

inoculating the culture medium with a desired cellular concentration of the microorganism;

aerating the culture medium with oxygen, wherein the process has a maximum oxygen supply rate to the culture medium;

supplying the culture medium with an alternative oxidant source, other than oxygen, such that when the oxygen requirements for cellular respiration of the microorganisms within the culture medium is less than the maximum rate of oxygen supply to the culture medium, then the microorganisms will substantially utilize oxygen for cellular respiration, and when the oxygen requirements for cellular respiration of the microorganisms within the culture medium is greater than the maximum rate of oxygen supply to the culture medium, then a portion of the microorganisms within the culture medium will utilize the alternative oxidant source for cellular respiration;

maintaining the culture medium at a desired pH and temperature; and

allowing the culture medium to incubate for a time sufficient to produce a desired quantity of a biological product.

2. The process of claim 1, further comprising the steps of isolating and recovering said biological product from said culture media.

3. The process of claim 1, wherein the microorganism is selected from the group consisting of bacteria, yeasts, molds and archaea.

4. The process of claim 3, wherein the microorganism is a bacteria.

5. The process of claim 4, wherein bacteria is a facultative aerobe.

6. The process of claim 5, wherein the facultative aerobe is from a genus selected from the group consisting of *Pseudomonas, Paracoccus, Micrococcus, Klebsiella, Escherichia, Acidianus, Campylobacter, Wolinella, Desulfovibrio, Clostridium,* and *Proteus*.

7. The process of claim 6, wherein the genus is *Pseudomonas*.

8. The process of claim 7, wherein the species of the genus *Pseudomonas* is selected from the group consisting of *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas cruciviae, Pseudomonas boreopolis* and *Pseudomonas oleovorans*.

9. The process of claim 8, wherein the species of *Pseudomonas* is *Pseudomonas aeruginosa*.

10. The process of claim 1, wherein the carbon substrate is selected from the group consisting of fatty acids, glycerol, low molecular weight acids, carbohydrates, yeast extract, peptone and vegetable oil.

11. The process of claim 10, wherein the fatty acids are selected from the group consisting of palmitic acid, stearic acid, oleic acid, linoleic acid, arachidic acid, butyric acid, caproic acid, lauric acid, and linolenic acid.

12. The process of claim 11, wherein the fatty acid is palmitic acid.

13. The process of claim 10, wherein the vegetable oil is selected from the group consisting of corn oil, peanut oil, coconut oil, linseed oil, olive oil, soy bean oil and sunflower oil.

14. The process of claim 13, wherein the vegetable oil is corn oil.

15. The process of claim 10, wherein the carbohydrate is glucose.

16. The process of claim 10, wherein the low molecular weight acid is selected from the group consisting of malate, acetate and pyruvate.

17. The process of claim 1, wherein the alternative oxidant source is selected from the group consisting of nitrates, nitrites, sulfates, sulfites, carbonates, fumarates, sulfur, manganic ion, ferric ion, selenate, dimethyl sulfoxide, arsenate, trimethylamine-N-oxide and glycine.

18. The process of claim 17, wherein the alternative oxidant source is a nitrate.

19. The process of claim 18, wherein the nitrate is selected from the group consisting of sodium nitrate, potassium nitrate, calcium nitrate, magnesium nitrate, ammonium nitrate, and nitric acid.

20. The process of claim 19, wherein the nitrate is sodium nitrate.

21. The process of claim 17, wherein the nitrites are selected from the group consisting of sodium nitrite, potassium nitrite, calcium nitrite, ammonium nitrite, and nitrous acid.

22. The process of claim 17, wherein the sulfates are selected from the group consisting of sodium sulfate, potassium sulfate, calcium sulfate, iron sulfate, magnesium sulfate, ammonium sulfate, zinc sulfate, copper sulfate, cobalt sulfate, manganese sulfate, and dilute sulfuric acid.

23. The process of claim 17, wherein the sulfites are selected from the group consisting of calcium sulfite, sodium sulfite, potassium sulfite, iron sulfite, magnesium sulfite, ammonium sulfite, zinc sulfite, copper sulfite, cobalt sulfite and manganese sulfite.

24. The process of claim 17, wherein the carbonates are selected from the group consisting of calcium carbonate, sodium carbonate, and potassium carbonate.

25. The process of claim 17, wherein the bicarbonates are selected from the group consisting of calcium bicarbonate, sodium bicarbonate, and potassium bicarbonate.

26. The process of claim 17, wherein the fumarates are selected from the group consisting of disodium fumarate, sodium fumarate, dipotassium fumarate, potassium fumarate, and fumaric acid.

27. The process of claim 1, further comprising the step of adding a sufficient amount of a surfactant to said culture medium to facilitate the mass transfer of said carbon substrate into said culture medium.

28. The process of claim 1, further comprising the step of limiting an essential growth nutrient from the culture medium.

29. The process of claim 28, wherein the essential growth nutrient is selected from the group consisting of phosphorous, nitrogen, sulfur, calcium, magnesium and iron.

30. The process of claim 29, wherein the essential growth nutrient is phosphorous.

31. The process of claim 1, wherein said cellular concentration of said microorganism is from about 0.1 g/L to about 50 g/L.

32. The process of claim 1, wherein the concentration of the alternative oxidant source in the culture medium is in the range of from about 0.01 to about 10 g/L.

33. The process of claim 1, wherein the culture is maintained in a temperature range of about 20°C to about 40 °C.

34. The process of claim 1, wherein the culture is maintained in a pH range of about 4 to about 9.

35. A process for the preparation of biological products under anaerobic respiring conditions comprising:

selecting a microorganism that is capable of utilizing an alternative oxidant source other than oxygen for cellular respiration under anaerobic conditions;

providing a culture medium suitable for the growth of the microorganism, wherein the medium comprises at least one carbon source;

inoculating the culture medium with a desired cellular concentration of the microorganism;

supplying an alternative oxidant source other than oxygen to the culture medium;

maintaining the culture medium at a desired pH and temperature; and

allowing the culture medium to incubate for a time sufficient to produce a desired quantity of a biological product.

36. The process of claim 35, further comprising the steps of isolating and recovering said biological product from said culture media.

37. The process of claim 35, wherein the microorganism is selected from the group consisting of bacteria, yeasts, mold and archaea.

38. The process of claim 37, wherein the microorganism is a bacteria.

39. The process of claim 38, wherein the bacteria is selected from the group consisting of obligate anaerobes and facultative aerobes.

40. The process of claim 39, wherein the obligate anaerobe is from a genus selected from the group consisting of *Desulfovibrio, Desulfomonas, Desulfotomaculum, Desulfobulbus, Desulfococcus, Desulfobacter, Desulfosarcine, Desulfonema, Desulfurmonas, Thermoproteus, Pyrococcus, Thermococcus*, and *Shewanella*.

41. The process of claim 40, wherein the facultative aerobe is from a genus selected from the group consisting of *Pseudomonas, Paracoccus, Micrococcus, Klebsiella, Escherichia, Acidianus, Campylobacter, Wolinella, Desulfovibrio, Clostridium,* and *Proteus*.

42. The process of claim 41, wherein the genus is *Pseudomonas*.

43. The process of claim 42, wherein the species of the genus *Pseudomonas* is selected from the group consisting of *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas cruciviae, Pseudomonas boreopolis* and *Pseudomonas oleovorans*.

44. The process of claim 43, wherein the species of *Pseudomonas* is *Pseudomonas aeruginosa*.

45. The process of claim 35, wherein the carbon substrate is selected from the group consisting of fatty acids, glycerol, low molecular weight acids, carbohydrates, yeast extract, peptone and vegetable oil.

46. The process of claim 45, wherein the fatty acids are selected from the group consisting of palmitic acid, stearic acid, oleic acid, linoleic acid, arachidic acid, butyric acid, caproic acid, lauric acid, and linolenic acid.

47. The process of claim 46, wherein the fatty acid is palmitic acid.

48. The process of claim 45, wherein the vegetable oil is selected from the group consisting of corn oil, peanut oil, coconut oil, linseed oil, olive oil, soy bean oil and sunflower oil.

49. The process of claim 48, wherein the vegetable oil is corn oil.

50. The process of claim 45, wherein the carbohydrate is glucose.

51. The process of claim 45, wherein the low molecular weight acid is selected from the group consisting of malate, acetate and pyruvate.

52. The process of claim 35, wherein the alternative oxidant is selected from the group consisting of nitrates, nitrites, sulfates, sulfites, carbonates, fumarates, sulfur, manganic ion, ferric ion, selenate, dimethyl sulfoxide, arsenate, trimethylamine-N-oxide and glycine.

53. The process of claim 52, wherein the alternative oxidant source is a nitrate.

54. The process of claim 53, wherein the nitrate is selected from the group consisting of sodium nitrate, potassium nitrate, calcium nitrate, magnesium nitrate, ammonium nitrate, and nitric acid.

55. The process of claim 54, wherein the nitrate is sodium nitrate.

56. The process of claim 35, wherein the nitrites are selected from the group consisting of sodium nitrite, potassium nitrite, calcium nitrite, ammonium nitrite, and nitrous acid.

57. The process of claim 35, wherein the sulfates are selected from the group consisting of sodium sulfate, potassium sulfate, calcium sulfate, iron sulfate, magnesium sulfate, ammonium sulfate, zinc sulfate, copper sulfate, cobalt sulfate, manganese sulfate, and dilute sulfuric acid.

58. The process of claim 35, wherein the sulfites are selected from the group consisting of calcium sulfite, sodium sulfite, potassium sulfite, iron sulfite, magnesium

sulfite, ammonium sulfite, zinc sulfite, copper sulfite, cobalt sulfite and manganese sulfite.

59. The process of claim 35, wherein the carbonates are selected from the group consisting of calcium carbonate, sodium carbonate, and potassium carbonate.

60. The process of claim 35, wherein the bicarbonates are selected from the group consisting of calcium bicarbonate, sodium bicarbonate, and potassium bicarbonate.

61. The process of claim 35, wherein the fumarates are selected from the group consisting of disodium fumarate, sodium fumarate, dipotassium fumarate, potassium fumarate, and fumaric acid.

62. The process of claim 35, further comprising the step of adding a sufficient amount of a surfactant to said culture medium to facilitate the mass transfer of said carbon substrate into said culture medium.

63. The process of claim 35, further comprising the step of limiting an essential growth nutrient from the culture medium.

64. The process of claim 63, wherein the essential growth nutrient is selected form the group consisting of phosphorous, nitrogen, sulfur, calcium, magnesium and iron.

65. The process of claim 64, wherein the essential growth nutrient is phosphorous.

67. The process of claim 35, wherein said cellular concentration of the microorganism in the culture medium is in the range of from about 0.1 g/L to about 50 g/L.

68. The process of claim 35, wherein the concentration of the alternative oxidant source in the culture medium is in the range of from about 0.01 to about 10 g/L.

69. The process of claim 35, wherein the culture is maintained in a temperature range of about 20°C to about 40 °C.

70. The process of claim 35, wherein the culture is maintained in a pH range of about 4 to about 9.

71. A process for increasing concentration of microorganisms in a defined medium comprising the steps of:

selecting a microorganism that is capable of utilizing oxygen or an alternative oxidant source other than oxygen for cellular respiration;

providing a culture medium suitable for the growth of the microorganism, wherein the medium comprises at least one carbon source;

inoculating the culture medium with a desired cellular concentration of the microorganism;

aerating the culture medium with oxygen, wherein the process has a maximum oxygen supply rate to the culture medium;

supplying the culture medium with an alternative oxidant source, other than oxygen, such that when the oxygen requirements for cellular respiration of the microorganisms within the culture medium is less than the maximum rate of oxygen supply to the culture medium, then the microorganisms will substantially utilize oxygen

for cellular respiration, and when the oxygen requirements for cellular respiration of the microorganisms within the culture medium is greater than the maximum rate of oxygen supply to the culture medium, then a portion of the microorganisms within the culture medium will utilize the alternative oxidant source for cellular respiration;

maintaining the culture medium at a desired pH and temperature; and.

allowing the culture medium to incubate for a time sufficient to produce a desired concentration of microorganisms within the culture medium.

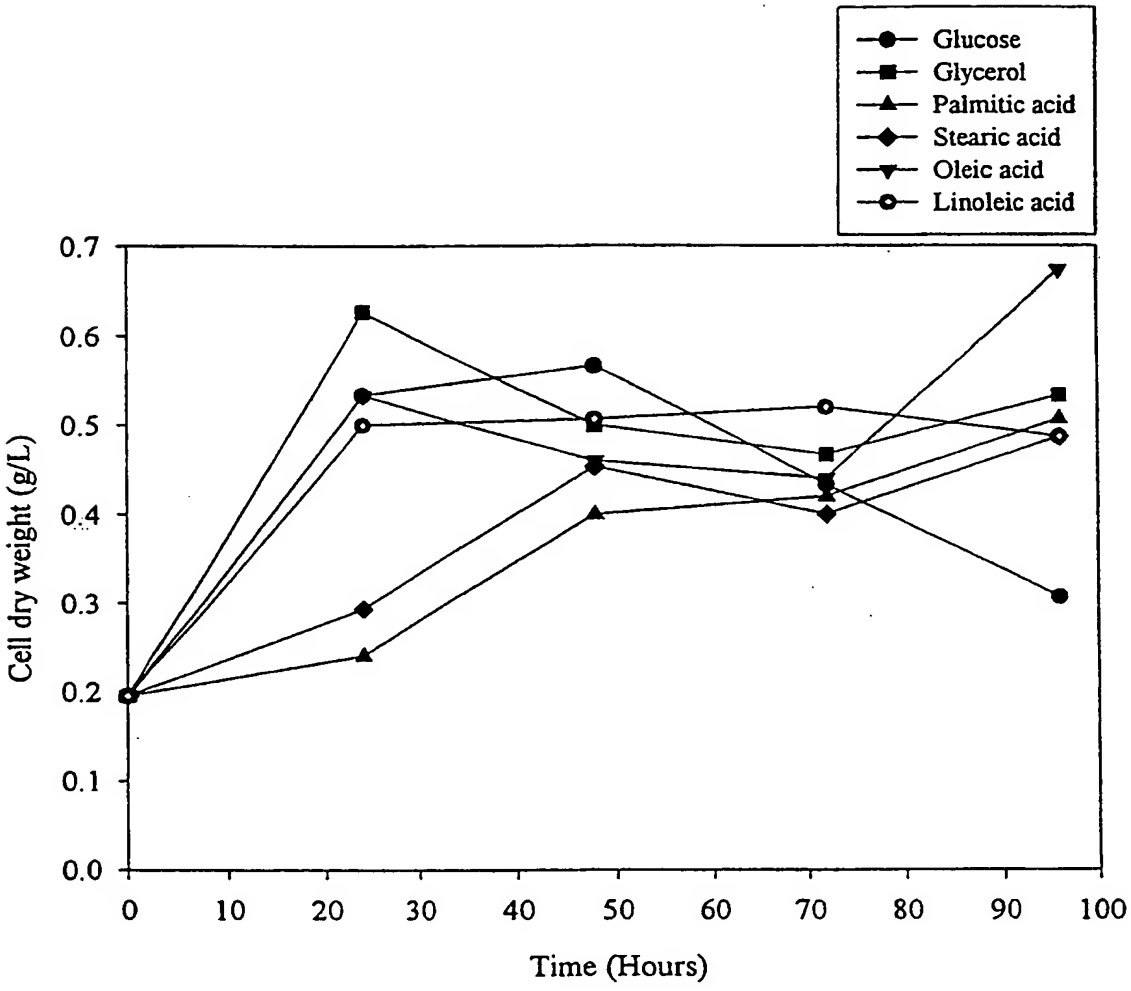

Figure 1

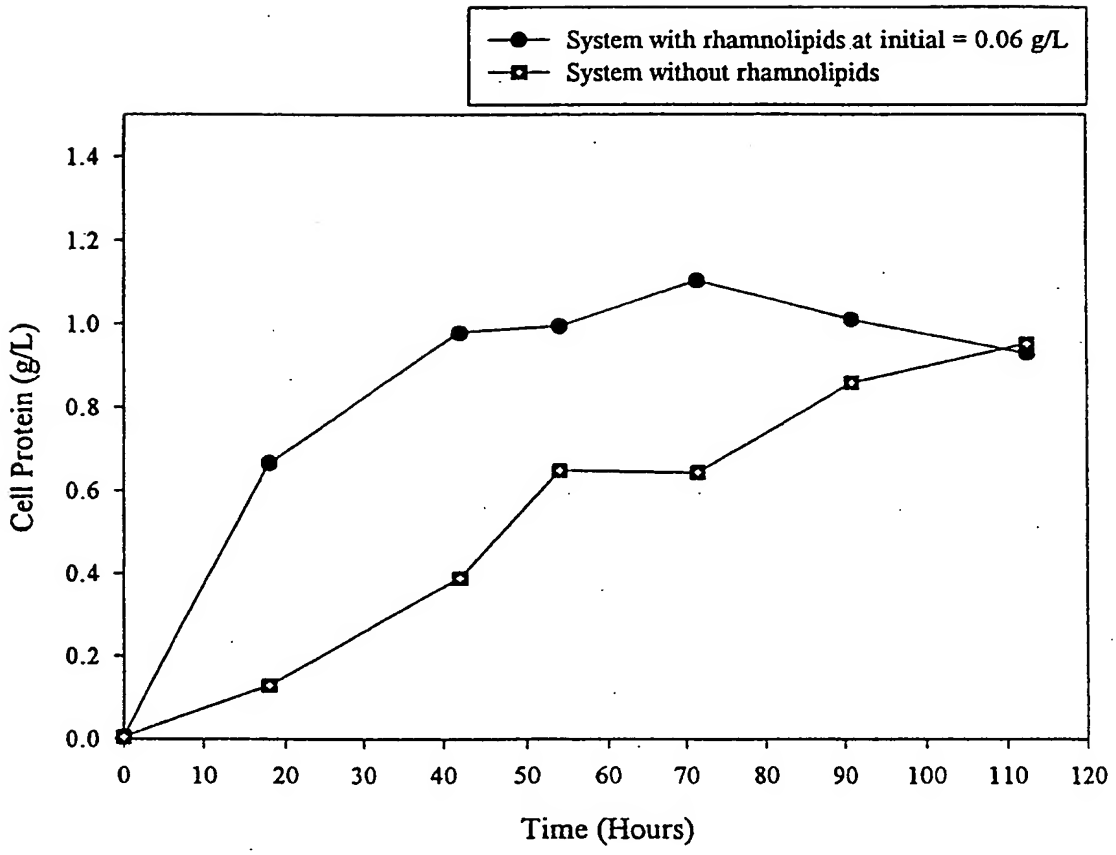
System with rhamnolipids at initial = 0.06 g/L
System without rhamnolipids
Cell Protein (g/L)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
80
90
100
110
120
Time (Hours)

Figure 2

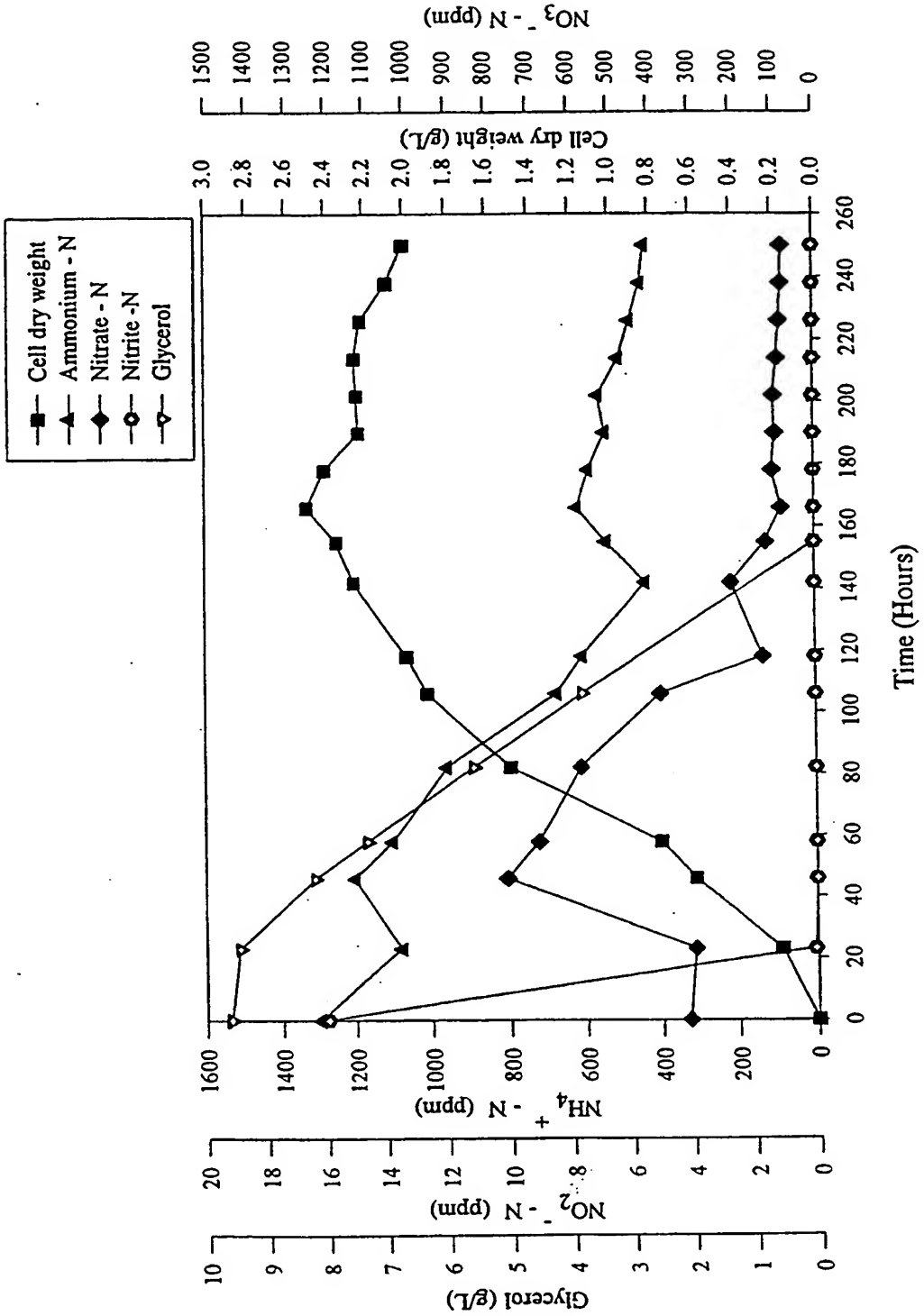

Figure 3

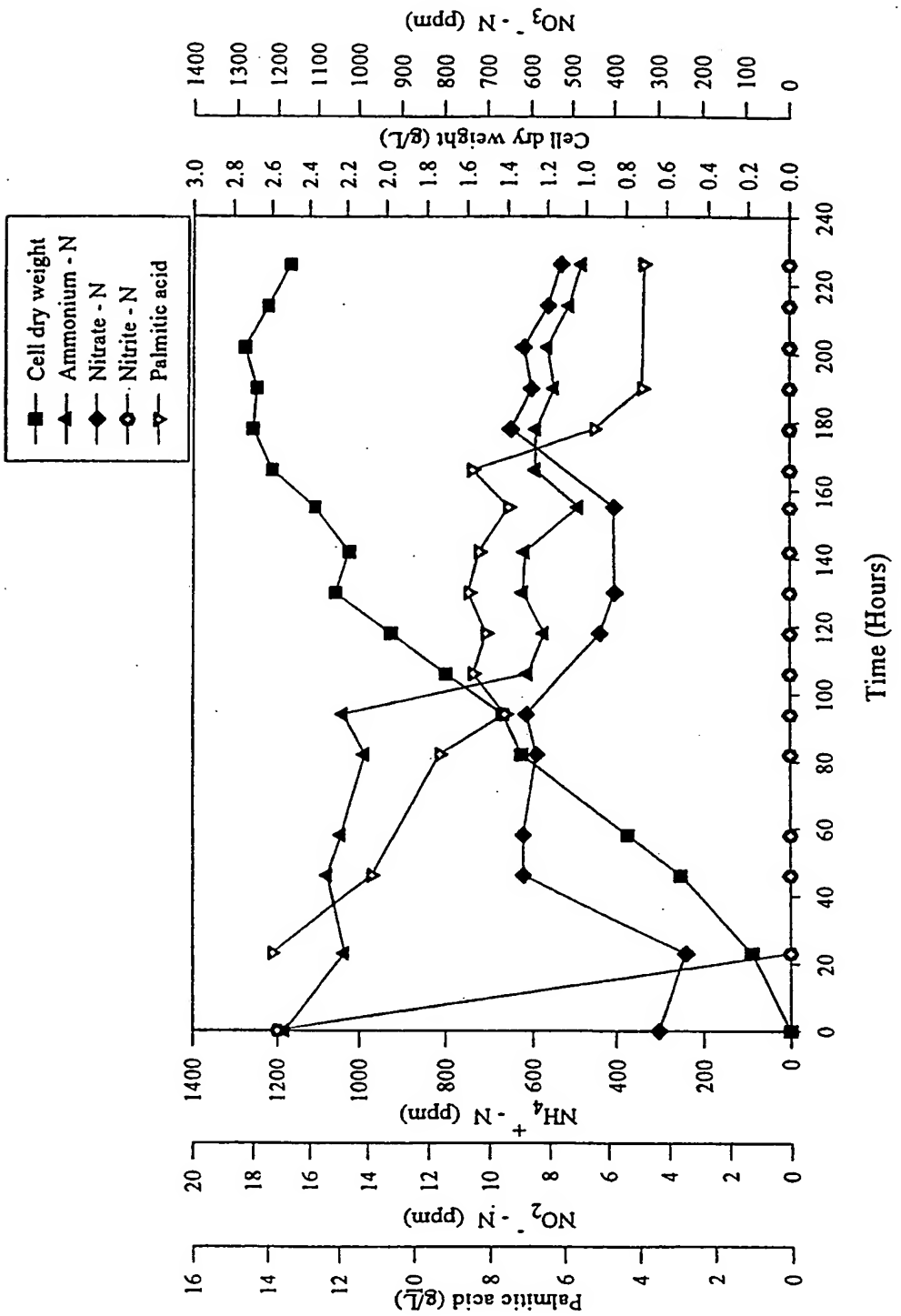
Cell dry weight
Ammonium - N
Nitrate - N
Nitrite - N
Palmitic acid
Time (Hours)
Palmitic acid (g/L)
NO_2^- - N (ppm)
NH_4^+ - N (ppm)
Cell dry weight (g/L)
NO_3^- - N (ppm)

Figure 4

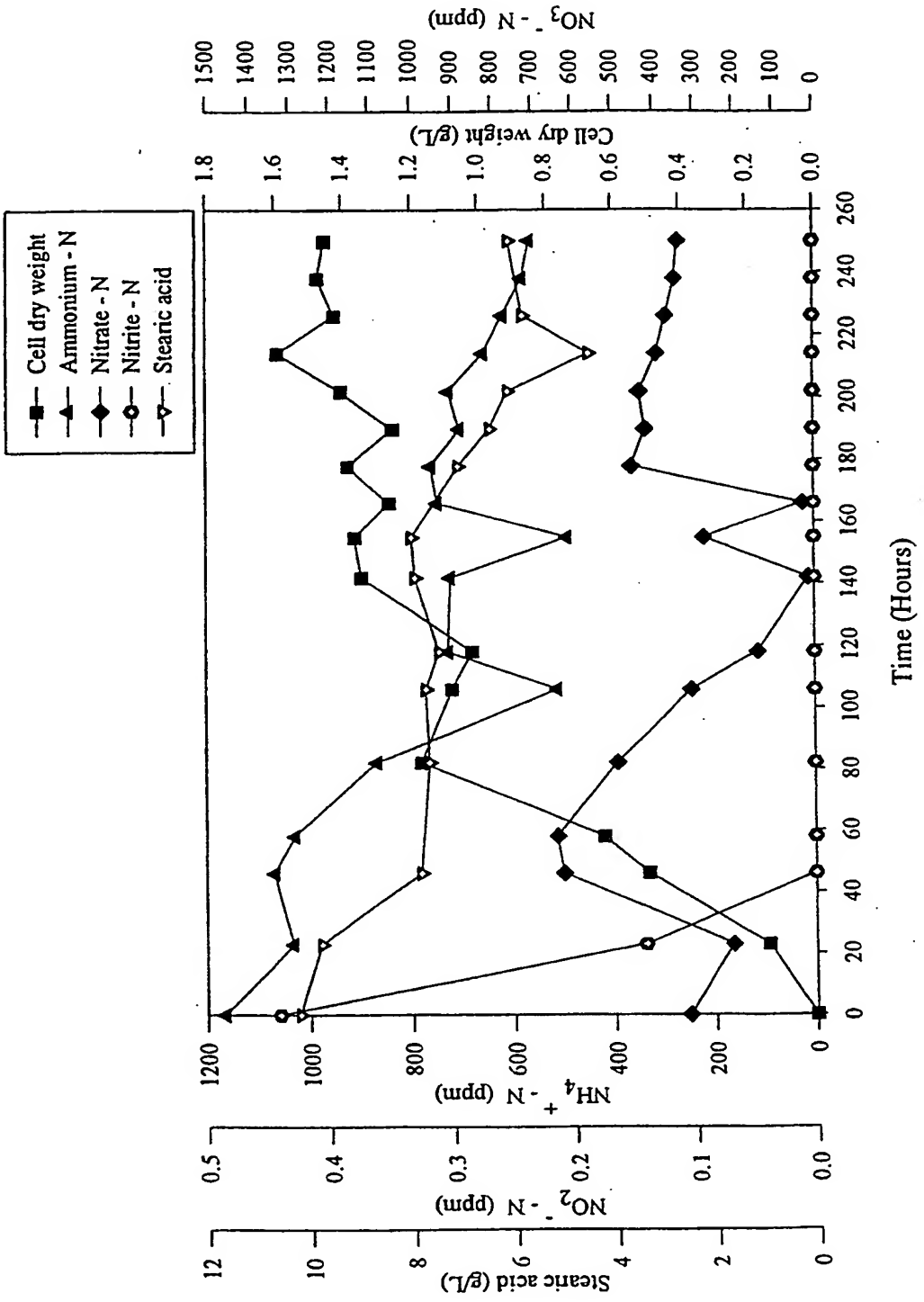

Figure 5

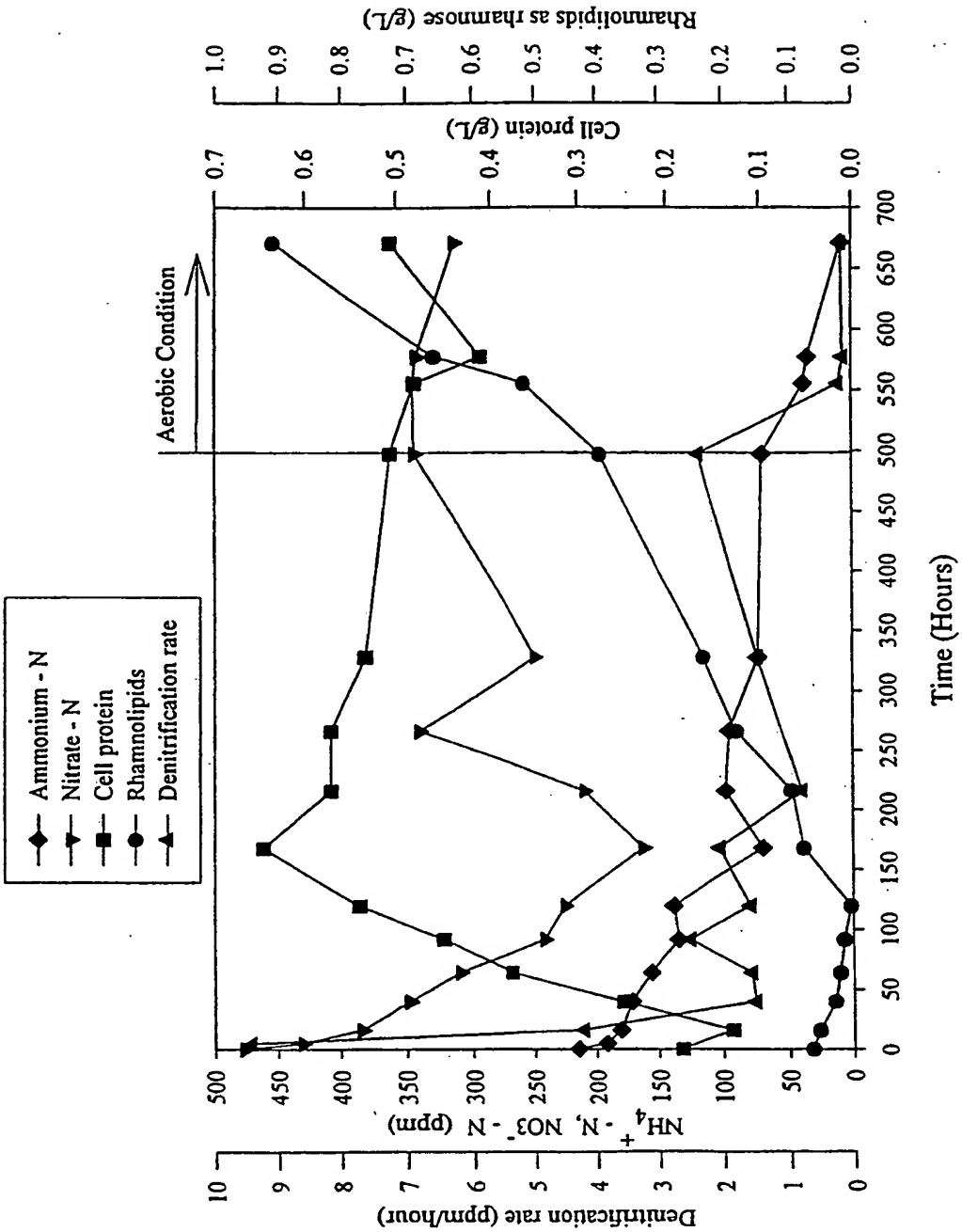

Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26950

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12P 1/00, 39/00, 19/02. 19/44; C12N 1/20

US CL :435/41, 42, 74, 105, 253.3; 536/4.4

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/41, 42, 74, 105, 253.3; 536/4.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US 5,501,966 A (GIANI ET AL) 26 MARCH 1996 (26/03/1996), see entire document, especially columns 3-9.	1-20,22-25, 27-38,41- 71 ---------- 21, 26, 39 and 40
Y	VARMA AMIT et al. Stoichiometric Flux Balance Models Quantitatively Predict Growth and Metabolic By-Product Secretion in Wild-Type Escherichia coli W3110. Applied and Environmental Microbiology.October 1994, Vol. 60, No. 10, pages 3724-3731, see entire document.	1-71

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 11 FEBRUARY 2000	Date of mailing of the international search report 14 APR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PADMA BASKAR Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992) ★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26950

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ATLAS RONALD. Hand Book of Microbiological Media. Ann Arbor: CRC press. 1993, pages 290-301, see entire document.	21,26,39 and 40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/26950

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

STN, WEST, HCAPLUS, BIOSIS, MEDLINE, EMBASE, LIFESCI, SCISEARCH, WPIDS.
search terms: anaerobic, aerobic, facultative aerobe , pseudomonas, obligate anaerobe, desulfomonas, desulfobacter, culture, media.